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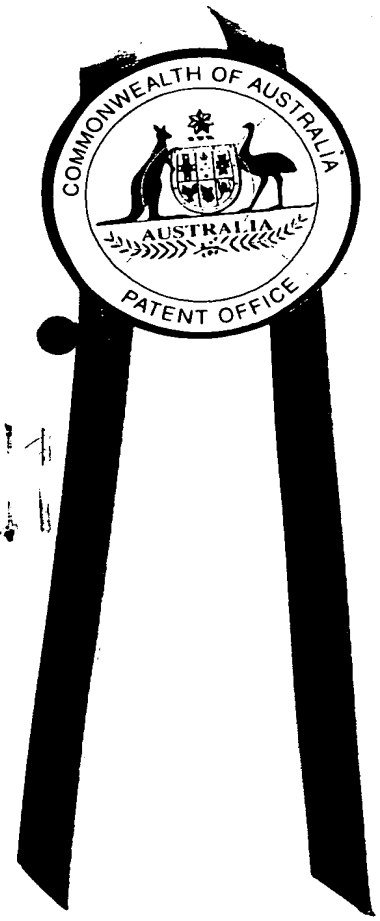

WITNESS my hand this
Eleventh day of May 2000

A. M. Madl

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PRIORITY DOCUMENT
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S & F Ref: 447430

ORIGINAL

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION FOR THE INVENTION ENTITLED:

Isoflavone Metabolites

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Name of Inventor: George Eustace Joannou

This invention is best described in the following statement:

derivatives circulate around the body and are mainly excreted in the urine, in which they can then be detected.

As stated above, given the presence of high levels of isoflavones in legumes, particularly soya beans, and the knowledge that the isoflavones are fermented or metabolised by intestinal or bowel bacteria to produce isoflavone metabolites, research has been conducted into microbial fermentations of soybeans and has demonstrated production of metabolites including 6,7,4'-trihydroxyisoflavone (hereinafter called Factor 2) and other polyhydroxylated isoflavonoids.

Traditional Asian food products such as tempeh, tofu, miso etc are foods produced from soybeans by fermentation mainly by fungi of the genus *Rhizopus*. It has been shown that several bacteria species may also be involved in tempeh production. For traditional tempeh fermentation, the soybeans are cooked, dehulled and soaked overnight. A spontaneous bacterial acidification occurs during this phase. In industrial tempeh fermentation processes, the cooked soybeans are acidified with lactic acid. After the soaking process, the soybeans are cooked again and incubated with microbial inocula for 2 days.

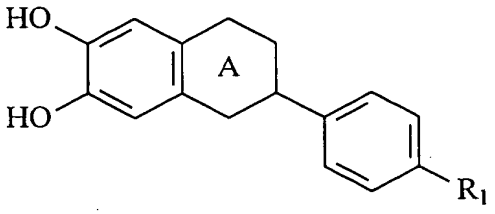
In unfermented soybeans, the isoflavones genistein, daidzein and glycitein predominantly occur as isoflavone glucosides and acylglucosides. It has been shown that during tempeh fermentation, the isoflavone aglycones are liberated from the conjugates and accumulate in the tempeh product. Further findings have shown that during fermentation the isoflavone 6,7,4'-trihydroxyisoflavone (termed "Factor 2" by Gyorgy *et al.* in Nature (1964) 203, 870-872), also accumulates.

It was previously thought that the fungi of the genus *Rhizopus* were responsible for the formation of Factor 2 from either daidzein or glycitein. However, subsequent studies on the metabolism of daidzein and glycitein by Klus *et al.*, 1993 showed that isolates of *Brevibacterium epidermidis* and *Micrococcus luteus*, which were isolated from Indonesian tempeh samples, readily transform glycitein, forming Factor 2. A third tempeh-derived bacterium, *Microbacterium arborescens*, metabolized daidzein, producing both Factor 2 and glycitein. More recently, Klus, K. and Barz, W. (1995) *Arch Microbiol* 164:428-434, investigated five other bacterial isolates, which were isolated from tempeh samples containing Factor 2 and were classified as *Micrococcus* or *Arthrobacter* strains, for their ability to metabolize daidzein and glycitein by hydroxylation or O-demethylation reactions. Their results show that a number of polyhydroxylated isoflavones were formed, hydroxylated at three or four of positions 6,7,8, 3' and 4'. Of these Factor 2 was the major product produced by most of the microbial strains. The bacterial strains only hydroxylated but did not degrade the substrates namely daidzein or glycitein. The compounds of the present invention were not identified by Klus and Barz, however,

Various polyhydroxylated isoflavones known in the prior art are known to exhibit anti-inflammatory and anti-allergenic activity and to express anticarcinogenic

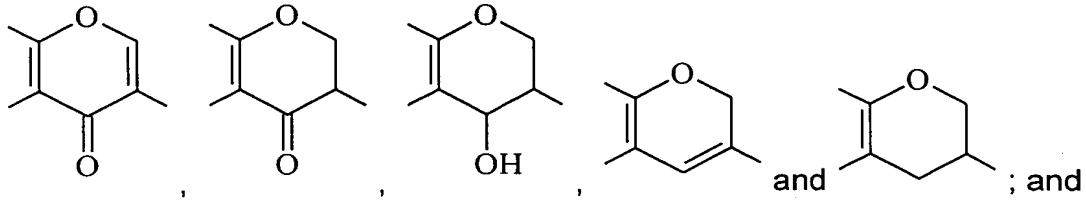
Summary of the invention

According to a first aspect of the present invention there is provided a compound of formula I



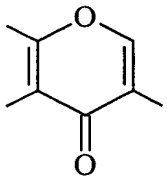
in which

A is selected from the group consisting of:



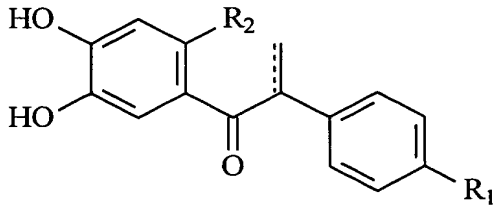
; and

R_1 is H, OH or OCH_3,



provided that when A is , R_1 is not OH.

According to a second aspect of the present invention there is provided a compound of Formula II



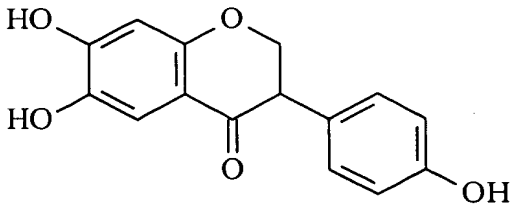
in which

R_1 is H, OH or OCH_3,

R_2 is OH or OCH_3, and

----- denotes a single or double bond.

A preferred compound of formula I is 4',6,7-trihydroxyisoflavanone having the structure:



According to a related sixth aspect of the present invention there is provided a method for the treatment, prophylaxis, amelioration, defence against, and/or prevention of hormone-dependent conditions including hormone dependent cancers such as breast cancer, hormone dependent cardiovascular disorder and hormone dependent menopausal disorders comprising administering to a subject a therapeutically effective amount of one or more compounds of the formulae I or II as previously defined, either alone or in association with one or more pharmaceutically acceptable carriers, diluents, adjuvants and/or excipients.

Typically, one or both of compounds A and B may be used in the method of treatment, prophylaxis, amelioration, defence against, and/or prevention of any one or more of the diseases of the fifth or sixth aspects of the invention.

A seventh aspect of the present invention is the use of one or more compounds of the formulae I or II for the manufacture of a medicament for the treatment, amelioration, defence against, prophylaxis and/or prevention of one or more of the diseases set out in the fifth or sixth aspects of the invention above.

It is typical that one or both of compounds A and B are employed in the seventh aspect of the present invention.

A related eighth aspect of the present invention is use of one or more compounds of the formulae I or II in the treatment, amelioration, defence against, prophylaxis and/or prevention of one or more of the diseases set out in the fifth or sixth aspects of the invention above.

Typically, one or both of compounds A and B are used in the eighth aspect of the invention.

A ninth aspect of the present invention is a microbial culture or a food or drink composition containing at least one microbial strain which microbial strain is capable of producing one or more compounds of the formulae I or II from daidzein and/or glycitein.

Typically, said microbial strain produces one or both of compounds A and B.

Typically, the microbial strain is in the form of a purified culture, which may optionally be admixed and/or administered with one or more other cultures which produce any one or more compounds of the formulae I or II, more typically one or both of compounds A and B.

A tenth aspect of the present invention provides a process for producing a compound of any one of formulae I or II by microbial fermentation of daidzein or glycitein. Usually, the microbial fermentation utilises one or more microbial organisms selected from the group consisting of *Lactobacilli*; *Clostridium perfingens*; Bacteroids including *B.vulgatus, B. thetaiotaomicron, B. distasonis*; *Candida albicans* and other yeast; Anaerobic cocci including *Ruminococcus*, *Eubacterium*, *Peptostreptococcus* (such as *P. productus* found in stools), *Clostridium*, *Bifidobacteria* (such as *B. adolescentis*, *B. infantis*, and *B. longum*),

diseases; atherosclerosis; premenstrual syndrome, including fluid retention, cyclical mastalgia, and dysmenorrhoea; coronary artery spasm; vascular diseases including Reynauds Syndrome; Buergers Disease; migraine headaches; hypertension; benign prostatic hypertrophy; all forms of cancer including breast cancer, endometrial cancer, prostatic cancer, uterine cancer, ovarian cancer, testicular cancer, large bowel cancer; Alzheimers disease; inflammatory diseases including Crohns disease, inflammatory bowel disease, ulcerative colitis; baldness including male pattern baldness; psoriasis; acne; and diseases associated with oxidant stress including myocardial infarction, sunlight induced skin damage, arthritis, or cataracts.

A fourteenth aspect of the invention further provides the use of Factor 2 for the manufacture of a medicament for the treatment, prophylaxis, amelioration, defence against, and/or prevention of hormone-dependent conditions including hormone dependent cancers such as breast cancer, hormone dependent cardiovascular disorder and hormone dependent menopausal disorders.

A fifteenth aspect of the present invention provides a process for the manufacture of Compound A, said process including:

i) mixing 2-(p-methoxyphenyl)propionic acid with 1,3,4-trimethoxy benzene to obtain 2,4,5,4'-tetramethoxy-α-methyldesoxybenzoin; and

ii) demethylating said 2,4,5,4'-tetramethoxy-α-methyldesoxybenzoin to form 2,4,5,4'-tetrahydroxy-α-methyldesoxybenzoin.

A sixteenth aspect of the present invention provides a compound when produced by the process of the fifteenth aspect of the invention outlined above.

The present invention is based upon the identification of novel oestrogenic isoflavone metabolite compounds, exemplified by two isoflavonoid phytoestrogens, which are termed Compounds A and B in the present specification. These compounds have been identified in the urine of the human adult consuming a diet rich in phytoestrogen content. While not wishing to be bound by theory, it is postulated by the present inventor that the identification of Compounds A and B provides evidence for the existence of a previously undiscovered pathway in the mode of metabolism of daidzein and/or glycitein.

The identification of compounds A and B observed for the first time in the urine of adult humans who ingested soya cake containing daidzein, genistein and glycitein provides evidence to suggest that compounds A and B are products of microbial transformations of daidzein or glycitein. In view of the fact that one of these metabolites, namely compound A, was found in large amounts commensurate to the amount of daidzein ingested compared with glycitein appears that compounds A and B may also be metabolites of daidzein after hydroxylation of ring A. The recent results of Klus K *et al.*, 1995 support this hypothesis since recently they have demonstrated that a number of microbial species (*Micrococcus*,

A.hydrophila; *Alcaligenes sp*; *Citrobacter sp*; *Enterobacter sp* including *E. liquefaciens* and *E. aerogenes*; *Escherichia sp*, *E coli*; *Hafnia sp*; *Klebsiella sp*; *Morganella sp* such as *M.morganii*; *Proteus sp*; *Pseudomonas sp*; *Providencia sp*; *Aerococcus viridans*; *Bacillus sp*; *Corynebacterium sp*; *Micrococcus sp* such as *M. luteus*; *Nocardia sp*; *Pediococcus sp*; *Staphylococcus sp* including *S aureus* and *S. epidermidis*; *Fusobacterium* including *F. gonidiaformans*, *F. mortiferum*, *F. necrogenes*, *F. necroforum* and *F. russii*; *Butyrivibrio* such as *B. fibrisolvens*; *Actinomyces, Arachnia-Propionibacterium; Arthrobacter sp* such as *A. agilis, A. aurescens, A. pascens, A. oxydans, A. nicotinae and A. cummins; Breribacterium sp* such as *B. epidermidis; and Microbacterium sp* such as *M. arborescens.*

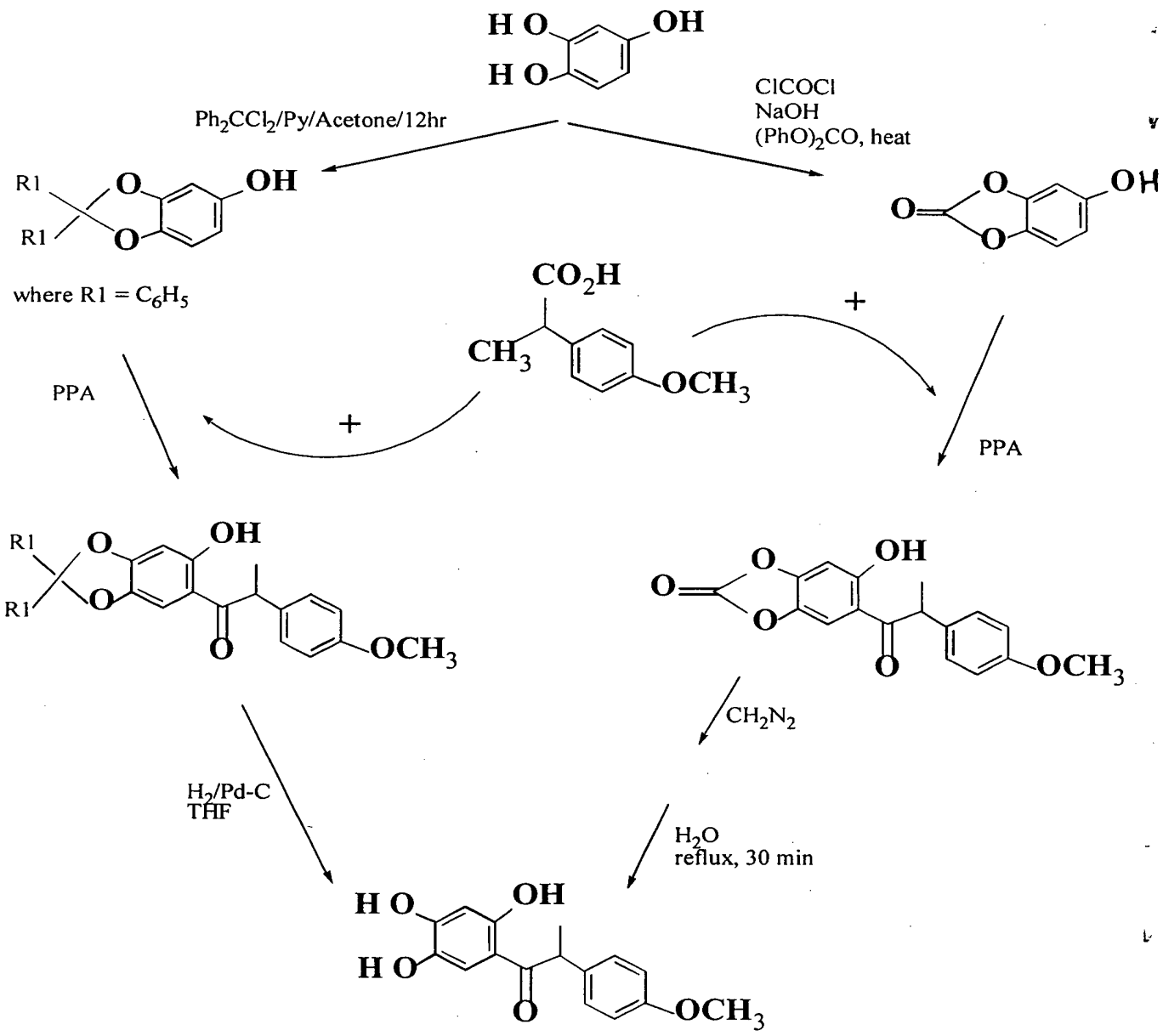
Typically, non-pathogenic organisms selected from the above organisms such as *Micrococcus sp* and *Arthrobacter sp* may be used directly in food and/or drink compositions such as dairy formulations so as to provide compounds of the formulae of the invention. The drink/food compositions also need to contain a plytoestrogen source such as soya.

Microbial Conversion of Daidzein and Glycitein to Factor-2 can be effected using the following microbial organisms: Arthrobacter including *agilis*, *aurescens*, *pascens*, *oxydans*, *nicotinae*, and *cumminsii*; Brevibacterium *epidermidis* (converts glycitein to Factor 2); *Micrococcus luteus* (converts glycitein to Factor 2), *Microbacterium arborescens* (converts daidzein to Factor 2 & glycitein), *Streptomyces sp roseolus* (converts daidzein/glycitein to 8,3'-dihydroxy-6,7,4-trimethoxyisoflavone or daidzein/glycitein to 7,8,4' & 7,3'4'-trihydroxyisoflavones, depending on culture medium). The various microbial conversions are disclosed in detail in Klaus, K. & Barz, W.: Arch Microbiol 164 (1995) 428-434; Klaus, K., Gabriele Borger-Papendorf & Wolfgang Barz: Biochemistry 34(4) (1993) 979-981; Mackenbrock, K and Barz W: Naturforsch 38c (1983) 708; Chimura, H. *et al*: J.Antibiot 28 (1975) 619-626; Funayama, S. *et al*: J.Antibiot 42(1989)1350-1355 and Komiyama, K. *et al*: J.Antibiot 42 (1989) 1344-1349, the contents of all of which are incorporated herein by reference.

An alternative source of compounds of the present invention, is that they can be obtained by chemical synthesis. Conveniently, Factor 2 or a naturally-occurring isoflavone such as glycitein may be utilised as starting material. Figures 2A and 2B demonstrate possible synthesis pathways of the compounds of the invention utilising glycitein as the starting material.

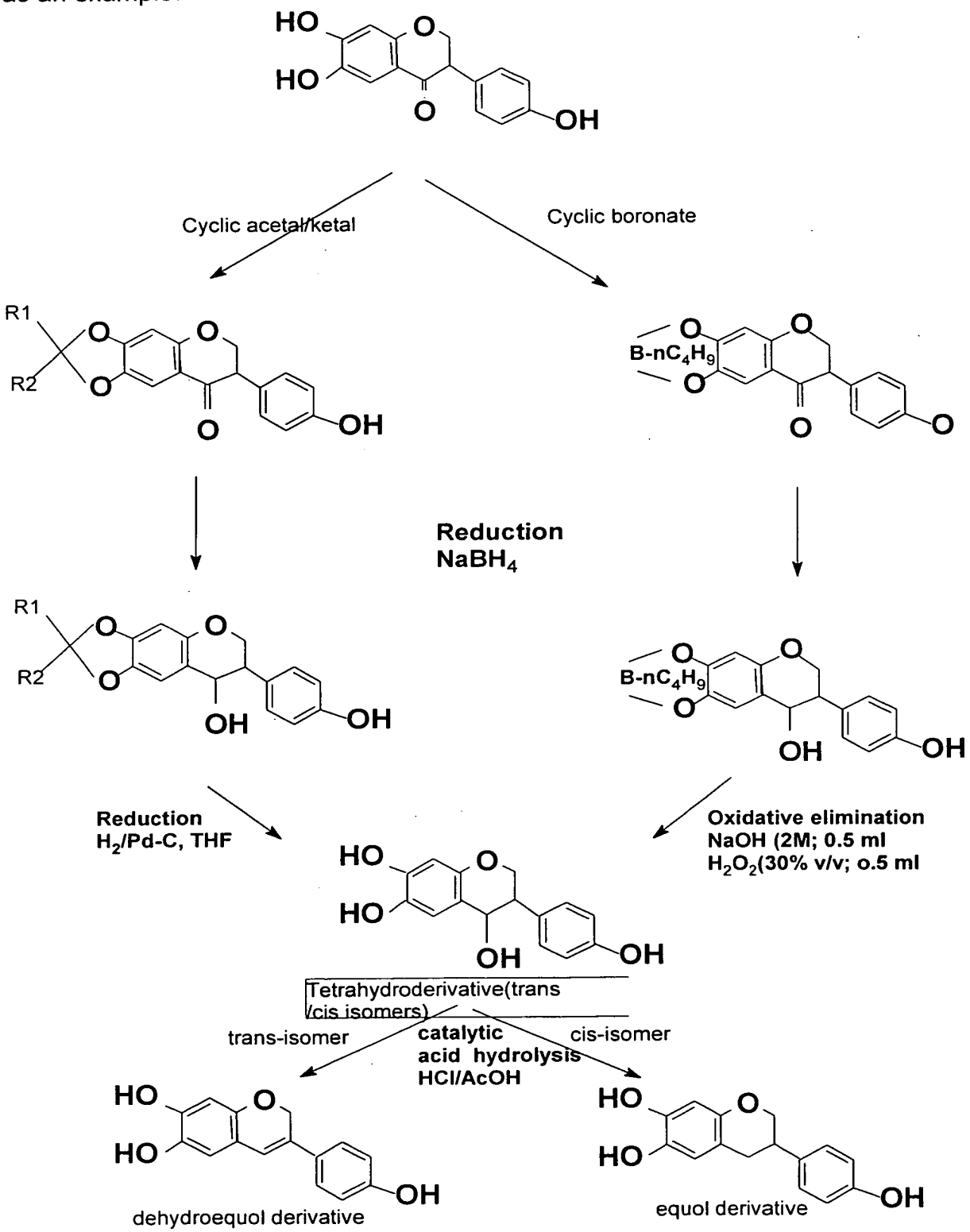
3 of Greene, T.W and Wuts, P.G.M : Protective Groups in Organic Synthesis (2nd Edition) (1991) John Wiley & Sons, Inc. USA; the disclosure of which is incorporated herein by reference.

Cyclic Carbonates, Acetals or Ketals



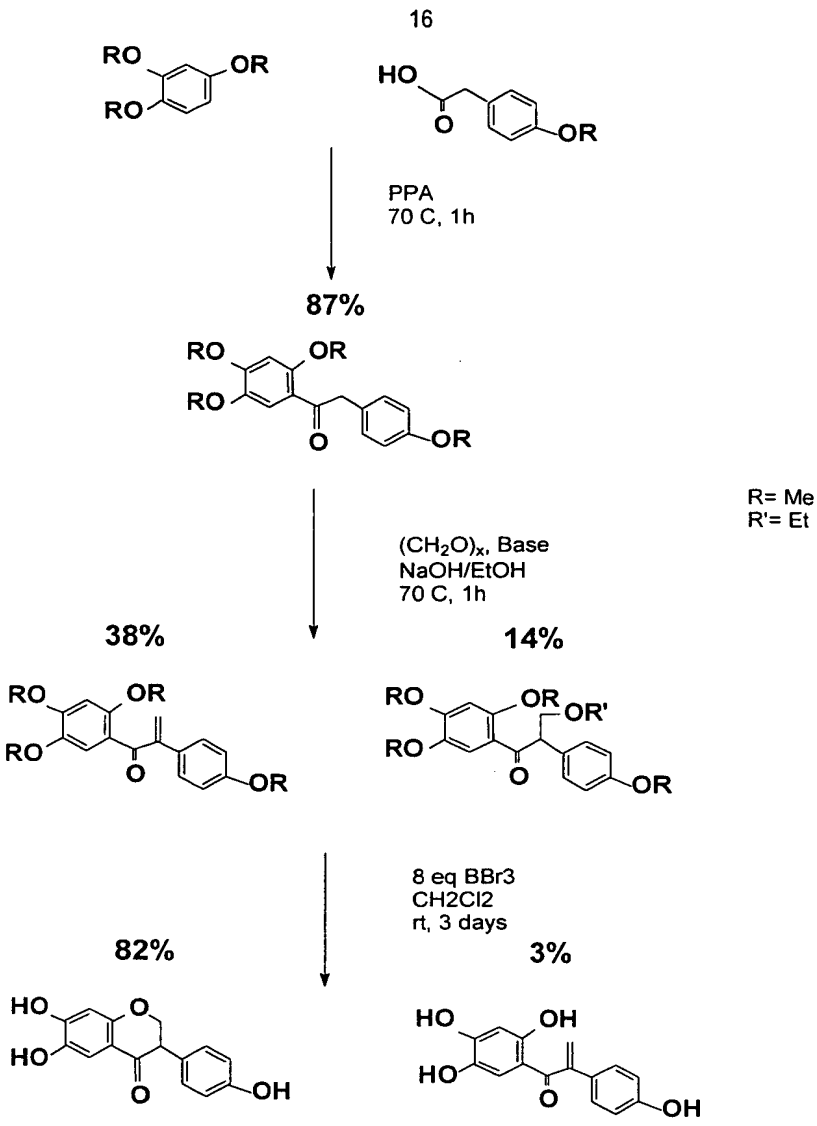
Scheme 2A

schematic representation (Scheme 3) is given below using 4',6,7-trihydroxyglycitein as an example.



Scheme 3

When necessary, hydroxyl groups in the compounds shown in Schemes 1-3 above, may be methylated and/or protected and deprotected, to give other



Scheme 5

In the above Scheme, the base is typically an organic amine, such as dimethylamine, or an alkali metal hydroxide, carbonate or bicarbonate. Best results have been obtained using 1% aqueous potassium bicarbonate.

In the synthesis of 4',6,7-trihydroxyisoflavone (5-deoxydihydroglycitein) shown in Scheme 5 above, the two intermediates obtained in the penultimate step prior to the demethylation with BBr_3 are not easily separated. However, it was found that a simple recrystallization procedure using methanol/water provided a quick method of separation and purification of the two intermediates. A similar procedure may be applied to the isolation of the methylated precursors of daidzein and genistein, namely formononetin and biochanin A which are present in clover and soya. Complete methylation of formononetin and biochanin A may further enhance the process of recrystallization of these two isoflavonoid precursors. Isolated formononetin or its fully methylated analogue can be used as a substrate for the

For parenteral administration, the compound(s) of the invention may be prepared in sterile aqueous or oleaginous solution or suspension. Suitable non-toxic parenterally acceptable diluents or solvents include water, Ringer's solution, isotonic salt solution, 1,3-butanediol, ethanol, propylene glycol or polyethylene glycols in mixtures with water. Aqueous solutions or suspensions may further comprise one or more buffering agents. Suitable buffering agents include sodium acetate, sodium citrate, sodium borate or sodium tartrate, for example.

Compositions of the invention may be prepared by means known in the art for the preparation of compositions (such as in the art of preparing veterinary and pharmaceutical compositions) including blending, grinding, homogenising, suspending, dissolving, emulsifying, dispersing and where appropriate, combining or mixing of the compound(s) of any of Formulae I or II, or Factor 2 together with the selected excipient(s), carrier(s), adjuvant(s) and/or diluent(s).

Compositions formulated as suitable for oral administration may be presented in discrete units, such as capsules, lozenges, or tablets, each containing a predetermined amount of the preferred active compound; as a solution or a suspension in an aqueous or non-aqueous liquid; as a powder or granules; or as an oil-in-water or water-in-oil emulsion. By way of example, compressed tablets may be prepared by compressing any one or more compounds of formulae I or II, or Factor 2, in a free-flowing form, such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, and/or surface active/dispersing agent(s) Moulded tablets may be made by moulding, in a suitable machine, a powdered compound of any one of formulae I or II, or Factor 2, moistened with an inert liquid binder.

Solid forms for oral administration may contain pharmaceutically or veterinarily acceptable binders, sweeteners, disintegrating agents, diluents, flavourings, coating agents, preservatives, lubricants and/or time delay agents. Suitable binders include gum acacia, gelatin, corn starch, gum tragacanth, sodium alginate, carboxymethylcellulose or polyethylene glycol. Suitable sweeteners include sucrose, lactose, glucose, aspartame or saccharine. Suitable disintegrating agents include corn starch, methylcellulose, polyvinylpyrrolidone, xanthan gum, bentonite, alginic acid or agar. Suitable diluents include lactose, sorbitol, mannitol, dextrose, kaolin, cellulose, calcium carbonate, calcium silicate or dicalcium phosphate. Suitable flavouring agents include peppermint oil, oil of wintergreen, cherry, orange or raspberry flavouring. Suitable coating agents include polymers or copolymers of acrylic acid and/or methacrylic acid and/or their esters, waxes, fatty alcohols, zein, shellac or gluten. Suitable preservatives include sodium benzoate, vitamin E, alpha-tocopherol, ascorbic acid, methyl paraben, propyl paraben or sodium bisulphite. Suitable lubricants include magnesium stearate, stearic acid, sodium

Suitable such materials are cocoa butter, waxes, fats, glycerol, gelatin and polyethylene glycols. Suitable enemas may comprise agents as exemplified above with reference to forms for topical administration.

Suitably, an inhalation spray comprising a compound(s) of Formulae I or II or Factor 2 will be in the form of a solution, suspension or emulsion as exemplified above. The inhalation spray composition may further comprise an inhalable propellant of low toxicity. Suitable propellants include carbon dioxide or nitrous oxide.

The pharmaceutical composition may contain pharmaceutically acceptable binders, diluents, disintegrating agents, preservatives, lubricants, dispersing agents, suspending agents and/or emulsifying agents as exemplified above. The veterinary composition may contain veterinarily acceptable binders, diluents, disintegrating agents, preservatives, lubricants, dispersing agents, suspending agents and/or emulsifying agents as exemplified above.

The invention includes compositions which are used for topical application which may be a cream, ointment, paste, solution, emulsion, lotion, milk, jelly, gel, spray, aerosol, oil, stick, roll-on or smooth-on, wherein the active compound comprises up to about 90%, more typically 10%, by weight of the composition, even more typically from about 0.1% to about 5% by weight, for example 3.5% by weight, even more typically from 0.5% to 2% w/w, and the compositions include topically suitable carriers, diluents, excipients, adjuvants and other additives.

Illustrative of pharmaceutically or cosmetically topically acceptable carriers or diluents are demineralized or distilled water; saline solution; vegetable based oils such as peanut oil, safflower oil, olive oil, cottonseed oil, maize oil, sesame oil, arachis oil or coconut oil; silicone oils, including polysiloxanes, such as methyl polysiloxane, phenyl polysiloxane and methylphenyl polysiloxane; volatile silicones; mineral oils such as liquid paraffin, soft paraffin or squalane; cellulose derivatives such as methyl cellulose, ethyl cellulose, carboxymethylcellulose, sodium carboxymethylcellulose or hydroxypropylmethylcellulose; lower alkanols, for example ethanol or iso-propanol; lower aralkanols; lower polyalkylene glycols or lower alkylene glycols, for example polyethylene glycol, polypropylene glycol. ethylene glycol, propylene glycol, 1,3-butylene glycol or glycerin; fatty acid esters such as isopropyl palmitate, isopropyl myristate or ethyl oleate; polyvinylpyrrolidone; agar; carrageenan; gum tragacanth or gum acacia, and petroleum jelly. Typically, the carrier or carriers will form from 10% to 99.9% by weight of the composition.

butylene glycol monostearate; sorbitol and ethoxylated sorbitol esters of fatty acids such as polyoxyethylene sorbitan monostearate, polyoxyethylene sorbitan monolaurate, polyoxyethylene sorbitan monooleate, polyoxyethylene sorbitan distearate, polyoxyethylene sorbitan dilaurate, polyoxyethylene sorbitan dioleate, polyoxyethylene sorbitan tristearate, polyoxyethylene sorbitan trilaurate or polyoxyethylene sorbitan trioleate; long-chain alcohols such as lauryl, myristyl, stearyl, oleyl, cetyl or cetostearyl alcohol; polysaccharides such as starch and starch derivative, cellulose derivatives as exemplified above, agar, tragacanth, acacia and alginic acid; and steroidal derivatives such as lanolin alcohols or ethoxylated lanolin alcohols, and beeswax. Illustrative ionic surfactants include triethanolamine and amine soaps such as triethanolamine stearate; anionic soaps such as calcium or magnesium salts of stearic acid or palmitic acid; fatty alcohol sulphates, for example sodium lauryl sulphate; alkyl or aralkyl sulphonates such as sodium sulphosuccinates or sodium dodecylbenzenesulphonate; quaternary ammonium salts containing at least one long-chain alkyl group as N-substituent, for example stearyl trimethylammonium chloride, and phosphate esters of polyalkylene glycols. Typically, the emulsifier or emulsifiers will form from 0.1% to 99% by weight of the composition.

The topical compositions of the invention may further include a sunscreen. Suitable sunscreens include opacifiers such as titanium dioxide or zinc oxide; p-aminobenzoic acid, isobutyl p-aminobenzoate, glyceryl p-aminobenzoate, or N-substituted derivatives of p-aminobenzoic acid such as isoamyl p-dimethylaminobenzoate, pentyl p-dimethylaminobenzoate, octyl p-dimethylaminobenzoate or ethyl 4-[bis(2-hydroxypropyl)amino]benzoate; 2-hydroxy-1,4-naphthoquinone; octocrylene; octyl p-methoxycinnamate or 2-ethoxyethyl p-methoxycinnamate; salicylate esters such as octyl salicylate, homomenthyl salicylate or 2-[bis(2-hydroxyethyl)-amino]ethyl salicylate; oxybenzone and methyl anthranilate. Typically, the sunscreen or sunscreens will form from 0. 1% to 10% by weight of the composition.

Additionally, it will be understood that the topical compositions of the invention may include suitable colouring agents and/or perfumes well known in the art. Typical examples of suitable perfuming agents are provided in S. Arctander, "Perfume and Flavor Chemicals", Montclair, New Jersey, 1969.

Formulations suitable for transdermal administration are typically presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Such patches suitably contain at least one compound of formulae I or II, or Factor 2, preferably one or both of compounds A and B, as an optionally buffered aqueous solution of, for example, 0.1M to 0.5M

for three days according to Bannwart C *et al.*, (*Finn. Chem. Lett.* 1984, Vol 11, p 120). NMR and GC-MS data identified the resulting product as **Compound A**. In performing the GC-MS, a 30 metre SE30 capillary column was used with a temperature program of 200-230°C at increments of 2°C/min, and 230-280°C at increments of 10°C/min. The carrier gas was Helium.

2. **Compound A** obtained as a product of acylation reaction

Step 1: Formation of 2,4,5,4'-tetramethoxy-α-methyldesoxybenzoin. To a mixture of 2-(p-methoxyphenyl)propionic acid (0.20 g, 1.11 mmol) and polyphosphoric acid (5 gm), 1,3,4-trimethoxy benzene (0.186 g, 1.11 mmol, 0.166 ml) was added. The mixture was allowed to heat to 75°C while stirring for 6 hours. TLC (30% EtOAc:Hexane) and gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses confirmed the presence of two major products with MU values of 24.68 and 25.01 (ratio 1:4). Chromatography on silica column (30% EtOAc:Hexane) allowed the isolation of the two products. Product MU 24.92 was isolated as a crystalline low melting solid. NMR data and GC-MS data confirmed the above structure. A 42% and 11% yield was obtained for products MU 25.01 and MU 24.68 respectively.

Step 2: Formation of 2,4,5,4'-tetrahydroxy-α-methyldesoxybenzoin. The product 2,4,5,4'-tetramethoxy-α-methyldesoxybenzoin (MU 25.01; 0.063 g) obtained from Step 1 above was dissolved in anhydrous dichloromethane (30.0 mls) and boron tribromide (0.271 g, 1.08 mmol 1.1 ml) was added to the solution. The mixture was allowed to stir at room temperature for 24 hours under nitrogen. TLC (30% EtOAc:hexane) established the presence of a single product which on GC analysis as the persilylated trimethylsilyl ether gave a single peak at MU 26.01. After work-up with ice/water the product was extracted with diethyl ether, washed with water, dried and concentrated to give a crude yellow oil, which NMR and GC-MS data confirmed the structure of the product being that of *2,4,5,4'-tetrahydroxy-α-methyldesoxybenzoin*.

3. **Compound B** from acylation reaction

Compound B was obtained in a three step reaction as illustrated in Scheme 3, involving an acylation reaction (step 1), formation of an α-alkenyl ketone (step 2) and cyclisation/demethylation (step 3). In brief, 2,4,5-trimethoxyphenyl-4'-methoxybenzyl ketone was obtained as an intermediate in an acylation reaction using 1,2,4-trimethoxybenzene (5.9 mmol), 4-methoxyphenylacetic acid (5.9 mmol) and polyphosphoric acid (17 gm) after heating at 70°C for one hour with mechanical stirring. Potassium carbonate was then added to the reaction for another one and

Compound A: (Acetone-d6; 2.05 ppm) δ 1.39 (3H, d, J=7.2Hz, CH3), 4.62 (1H,q,J=7.2 Hz, CH), 6.29 (1H,s,ArH-3), 6.75 (2H, d, J= 9.2 Hz, ArH3',5'), 7.17 (2H,d,J=9.2 Hz, ArH2',6'), 7.33 (1H,s, Ar-6)8.73

^{13}C n.m.r.

Compound A: (Acetone-d6, ppm) 18.73, 45.59, 103.05, 110.845, 115.38, 115.58, 128.51, 132.96, 137.60, 153.86, 156.25, 159.85, 204.77.

UV: λ_{max} = 283 nm

Compound B: HR: 272.0673 theoretical 272.0673

EIMS: m/z (% rel int) 272 [M]+ (31), 244 (9); 168(7); 153 (100); 120 (40); 107 (27); 91 (11).

CIMS: 301 M+29 (14); 273 M+1 (52); 257 (37); 137 (23); 97 (17); 83 (45); 71 (100).

EIMS as the tri-trimethylsilyl derivative: MU 28.48. MW 488; 488 (14); 473 (7); 369 (30); 296 (100); 281 (9); 192 (27); 177 (24); 147 (9).

NMR Data

^{1}H n. m. (Acetone-d6, δ 2.05 ppm

(1H, dd, $J_{3,\ 2eq}$ = 5.0 Hz, $J_{3,2ax}$ = 9.5 Hz, H-3), 4.14 (1H, dd, $J_{2ax,2eq}$ = 9.7 Hz, J $J_{2ax,3}$ = 9.6 Hz, H $_{2ax}$), 4.99 (1H, dd, J $_{2eq,2ax}$= 9.8 Hz, J 2eq,3 = 4.9 Hz, H $_{2eq}$), 6.38 (1H, s, ArH-8), 6.82 (2H, d, J=8.6 Hz, ArH-3',5'), 7.27 (2H, d, J= 8.6Hz, ArH-2',6'), 7.46 (1H, s, ArH-5).

^{13}Cn.m.r. (Acetone-d6, 29.8 ppm) δ 33.5, 54.6, 103.9, 111.9,, 116.6, 128.4, 129.3, 138.78, 155.2, 158.0, 160.4, 201.8.

UV: λ_{max} = 284 nm

EXAMPLE 2

Bacterial sp and culture conditions:

The standard incubation assays of bacteria (100 mg wet wt) with isoflavone substrates (5 x 10^{-5} M), the composition of the mineral salt medium and the isolation of the transformation products from the medium were essentially as described according to Klus, K. *et al*, (1995). The mineral medium and micronutrients were used according to Pfennig and Lippert (1966). In summary Bacterial sp were cultivated on Merck Standard I nutrient agar and for incubation experiments for 15 hr in 100 ml Merck Standard I nutrient broth. Prior to incubation the bacteria were washed twice with 200 ml Kpi buffer (0.05M, pH 7.5). After centrifugation (10,000 g, 15 min) 100 mg bacteria (fr. Wt) were inoculated in 5 ml mineral medium and 50 µl substrate solution (DMSO-MeOH, 1:10) was applied to

some proliferative activity of cancer cells was demonstrated at concentrations of 0.025μg/ml, whereas 5-hydorxy-O-Dma showed no proliferative activity of cancer cells.

When daidzein, formononetin, biochanin A and other metabolites of daidzein and genistein such as dihydrodaidzein, tetrahydrodaidzein (transisomer), O-Dma, 6-hydroxy-O-Dma and equol were tested for their inhibitory effect on MCF7 cells, it was found that with the exception of biochanin A and 6-hydroxy-O-Dma which showed some inhibition with an IC50 of 18-23 μg/ml at 72 and 144 hours incubation, all other metabolites had no significant effect,with their IC50 values at about 36->50μg/ml.

These results suggest that compound A is a potent inhibitor of breast cancer cells but more importantly, compound A showed no proliferative activity of cancer cells at low concentrations as genistein does. The 6,7-dihydroxy groups in compounds of the invention appear to be critical for this difference of biological activity of compounds of the invention when compared with analogues such as O-Dma and 6-hydroxy-O-Dma.

EXAMPLE 5

Comparative inhibitory effects of daidzein and genistein, their methylated analogues and metabolites with 5-hydroxy-O-Dma (compound A) on breast cancer cells and on normal breast cells

A. 5-Hydroxy-O-Dma when tested with MDA-MB-468 (estrogen negative) cancer cells showed significant inhibition at day 6 (IC50 = 6.8 μg/ml) as compared with 8.8 μg/ml for genistein and 3-7 times more inhibitive when compared with analogues of daidzein and genistein namely O-Dma (20 μg/ml) and 6-hydroxy-O-Dma (43 μg/ml) respectively. The IC50 of 5-hydroxy-O-Dma using MCF-7 estrogen positive breast cancer cells on day 6 of incubation was 2.1 μg/ml for 5-hydroxy-O-Dma as compared with the analogues of daidzein and genistein namely O-Dma (38 μg/ml) and 6-hydroxy-O-Dma (33 μg/ml) respectively.

B. 5-Hydroxy-O-Dma and genistein were tested with MCF-10A (normal breast cells). While genistein showed inhibition at days 1, 2 and 3 with an IC50 of 12, 10 and 12 μg/ml, 5-hydroxy-O-Dma did not show any inhibition with IC50 values > 54 μg/ml.

These results suggest that inhibition of 5-hydroxy-O-Dma like that of genistein, was more severe for the estrogen negative (-ve) cancer than that of the estrogen positive (+ve) cancer cells which suggests that in both these cases the mechanism of action is not related to the estrogen receptors.

EXAMPLE 6

Inhibitory effects of Factor-2 on breast cancer cells

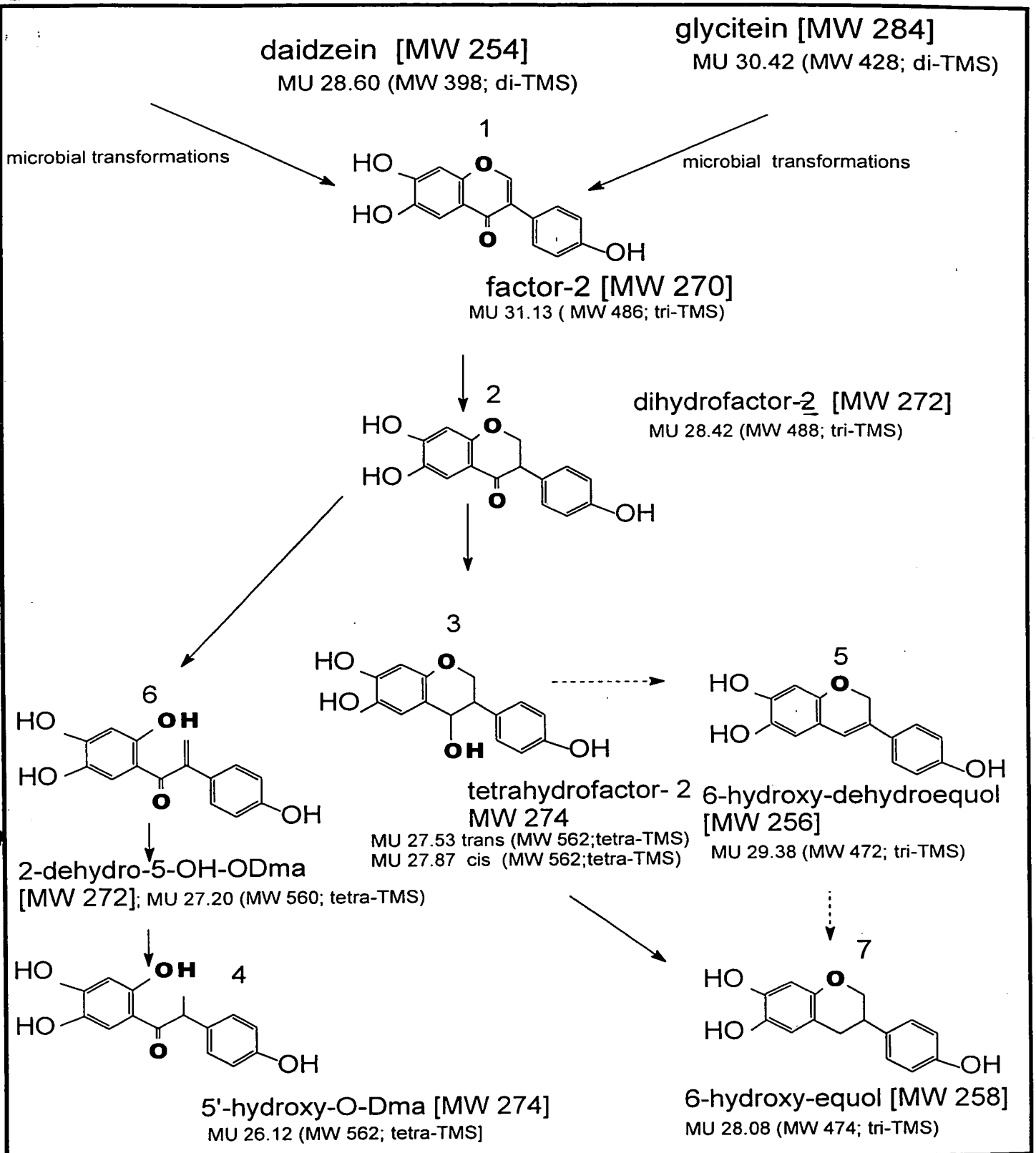
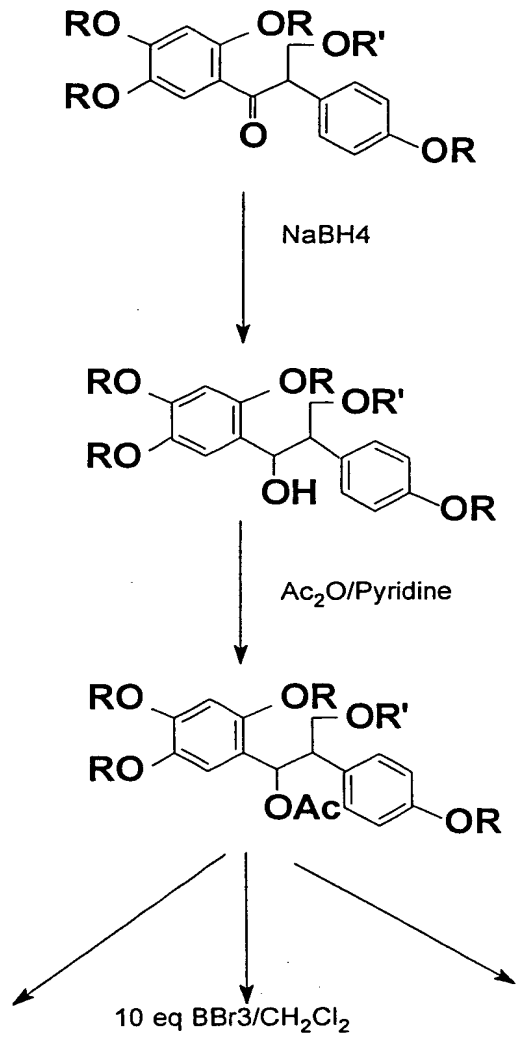
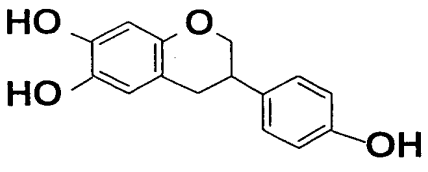

daidzein [MW 254]
MU 28.60 (MW 398; di-TMS)
glycitein [MW 284]
MU 30.42 (MW 428; di-TMS)
microbial transformations
microbial transformations
1
factor-2 [MW 270]
MU 31.13 (MW 486; tri-TMS)
2
dihydrofactor-2 [MW 272]
MU 28.42 (MW 488; tri-TMS)
3
tetrahydrofactor- 2
MW 274
MU 27.53 trans (MW 562;tetra-TMS)
MU 27.87 cis (MW 562;tetra-TMS)
5
6-hydroxy-dehydroequol
[MW 256]
MU 29.38 (MW 472; tri-TMS)
6
2-dehydro-5-OH-ODma
[MW 272]; MU 27.20 (MW 560; tetra-TMS)
4
5'-hydroxy-O-Dma [MW 274]
MU 26.12 (MW 562; tetra-TMS]
7
6-hydroxy-equol [MW 258]
MU 28.08 (MW 474; tri-TMS)

FIGURE 1

Synthesis of Compounds 3, 5 and 7 from intermediate 8

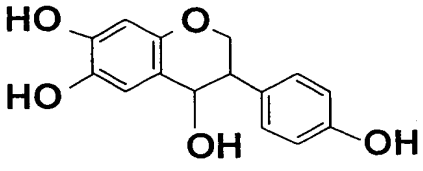


7



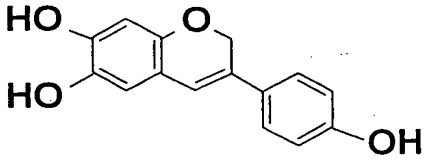
6-hydroxy-equol

3



tetrahydrofactor-2

5



6-hydroxy-dehydroequol

Figure 2B